Volume 313, number 1, 59–61 FEBS 11770 November 1992

Formation of a covalent Hg-Cys-bond during mercurial activation of PMNL procollagenase gives evidence of a cysteine-switch mechanism

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Received 1 October 1992

A common method for the activation of mammalian metalloproteinases is the use of mercurial compounds. Activation of PMNL procollagenase by soluble mercurials takes place as a three-step mechanism with a final intermolecular loss of the PRCGVPD autoinhibitor region. In this study covalently bound mercury in the form of mercurial agarose was chosen to probe activation of PMNL procollagenase. Activation was not achieved, since the final intermolecular cleavage with removal of the PRCGVPD motif could not take place. An intermediate form of the enzyme was bound to the column. Its N-terminal sequence determination proved cleavage of the Asp^{64}–Met^{65} peptide bond leaving the cysteine of the propeptide domain for covalent attachment to the mercurial agarose. This gives further evidence of a cysteine-switch mechanism involving Cys^{71}.

Collagenase; Neutrophil; Cysteine-switch mechanism; Activation

1. INTRODUCTION

Collagenase isolated from human polymorphonuclear leukocytes belongs to the metalloproteinase family [1], whose members share similar domain structures [2]. Its ability to cleave collagen types I, II, and III has been found to be important in processes such as leukodiapedesis and rheumatoid arthritis. All metalloproteinases are secreted as zymogens, and removal of an approximately 80 amino acids long propeptide is essential for activity. A zinc atom in the enzyme is believed to be complexed by two histidine residues and a third, yet unidentified amino acid from the catalytic domain [3]. According to the cysteine-switch model the fourth ligand is a cysteine residue from the conserved PRCGVPD region of the propeptide domain. Removal of this cysteine is assumed to be the crucial step in activation [4]. Recently Salow et al. have identified this zinc ligand in stromelysin-1 [5], which is another member of the metalloproteinase family.

In vitro activation can be attained by enzymes such as trypsin and cathepsin G [6] or by mercurials [7] and oxidative agents [8]. Mercurial activation has often been studied. Activation of PMNL collagenase by soluble mercurials has previously been found to take place in a three-step mechanism by successive intramolecular cleavage of the Asn^{53}–Val^{54}, Asp^{64}–Met^{65} and final loss of the PRCGVPD region by intermolecular cleavage of the Phe^{70}–Met^{80} or Met^{80}–Leu^{81} peptide bond. It has never been directly demonstrated that mercurials interact with the PRCGVPD autoinhibitor region of metalloproteinases [9]. In this paper we proved this modification during mercurial activation for the first time, which gives further evidence of a cysteine-switch mechanism.

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Abbreviation: PMNL, polymorphonuclear leukocytes.

2. MATERIALS AND METHODS

2.1. *Materials*

Dinitrophenyl-peptide was purchased from Bachem (Bubendorf, Switzerland). Affi-Gel-501 was from Bio-Rad (Richmond, USA). Plasmatonin was a kind gift of Fresenius (Oberursel, Germany).

2.2. *Purification of PMNL-procollagenase*

Procollagenase was purified as recently published [6]. Latency was scrutinized by degradation studies of synthetic octapeptide (Dnp-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-ArgOH) according to the method of Masui [10].

2.3. *SDS-PAGE*

SDS-PAGE was performed according to the method of Laemmli [11]. The protein was visualized by silver staining [12].

2.4. *Sequence determination*

Amino-terminal sequence determination was performed using a microsequencer (Model 810, Knauer, Berlin, Germany) as published before [13].

2.5. *Mercurial activation*

Activation by $HgCl_2$ was carried out as reported before [7].

2.6. *Activation of PMNL-procollagenase by mercurial agarose*

300 µl Affi-gel-501 [14] were washed several times with buffer (20 mM Tris-HCl pH 7.5; 10 mM $CaCl_2$; 0.0005% Triton X-100). 500 µl procollagenase (150 µg) in the same buffer was subjected to the gel material and continuously shaken at 37°C. After 4 h incubation the

reaction mixture was subjected to a column and rinsed with 10 mM EDTA. After washing 10 ml, the collagenase was eluted by 0.4 M mercaptoethanol, 1 mM EDTA. The EDTA eluate and the EDTA/mercapto eluate were collected and desalted by Bakerbond SPE-Wide-pore-Hi-Propyl column.

3. RESULTS AND DISCUSSION

Organomercurial agarose (Affi-Gel 501) has often been used to purify proteins which contain a free cysteine residue. Absorption to this material is due to the formation of covalent mercaptide bonds to the phenyl mercury resin. Elution of these proteins can be performed by mercaptoethanol or dithioerythritol.

PMNL collagenase shares its domain structure with fibroblast type collagenase and stromelysins I–III. They all consist of a propeptide and a catalytic domain followed by a hemopexin-like domain, which is coupled by a hinge region to the first two domains [15]. PMNL collagenase contains three cysteine residues, with two of them believed to be disulfide bridged in the hemopexin-like domain and one unbridged in the propeptide domain. Thus, mercurials are widely used to activate vertebrate metalloproteinases. Activation by oxidative agents and oxidised glutathione may occur in a similar manner and thus make mercurial activation a good model for studying this autoproteolytic mechanism.

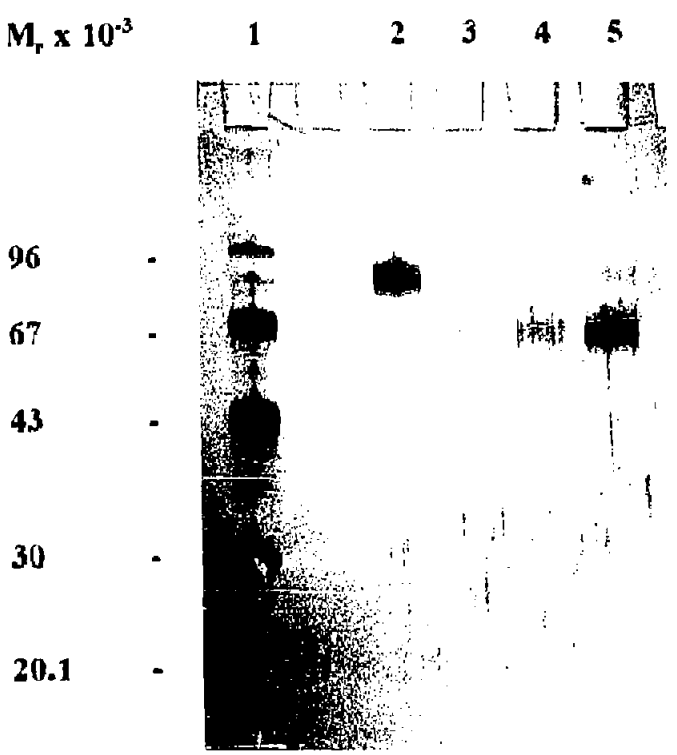

Fig. 1. SDS-PAGE, proteins visualized by silver staining. Lane 1, molecular weight marker; lane 2, latent PMNL collagenase; lane 3, supernatant after activation of collagenase by Affi-gel 501, lane 4, mercapto eluate of the resin after activation of PMNL collagenase; lane 5, collagenase activated by $HgCl_2$.

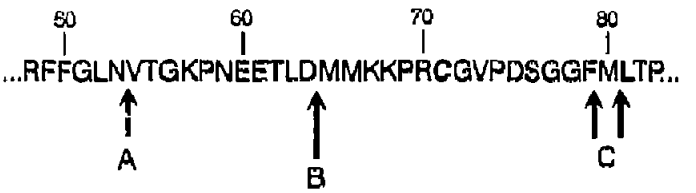

Fig. 2. Cleavage observed during activation of PMNL collagenase by soluble mercurials. A, first intramolecular cleavage; B, second intramolecular cleavage and N terminus of the enzyme after activation with mercurial agarose; C, N terminus of the fully active enzyme after the third intermolecular cleavage.

Until now experimental proof of an interaction of the mercury with the cysteine residue of the highly conserved PRCGVPD region is lacking. In order to test this hypothesis, we used Affi-Gel-501 for the activation of fully latent PMN leukocyte procollagenase. After 4 h incubation of the enzyme with the gel material, no enzyme was detected in the supernatant (Fig. 1) However, after concentration of the supernatant by a reversed-phase Hi-propyl column, several peptides of the propeptide domain could be identified by N-terminal sequence analysis. These exhibited proteolytic fragmentations yielding peptides beginning with Phe^{1}, Val^{54} and at several other sites of the propetide domain as published before for activation by $HgCl_2$ [7] (Fig. 2). Elution of the column by reducing buffer containing 0.4 M mercaptoethanol resulted in collagenase with an apparent molecular weight of M_r 67,000. N-terminal sequence determination of this protein revealed Met^{65} as the N-termi-

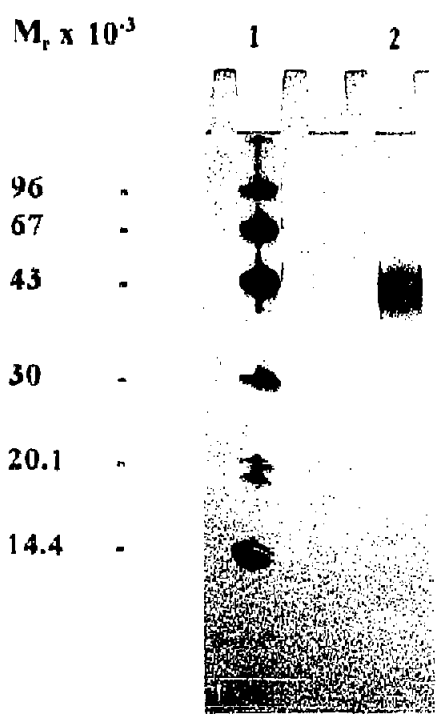

Fig. 3. SDS-PAGE, proteins visualized by silver staining. Lane 1, molecular weight marker; lane 2, collagenase fragment isolated after overnight incubation of collagenase with the resin, elution by reducing buffer and purification by reversed-phase HPLC.

nus indicating cleavage of the Asp^{64}–Met^{65} peptide bond in the proenzyme. Formation of this N terminus has previously been found to occur in a second step during the process of activation by soluble mercurials [7]. This might indicate that the two first cleavages are intramolecular events occurring after mercury-induced rearrangement of the initially cysteine-arrested propeptide domain. This partially truncated enzyme was not proteolytically active as demonstrated earlier [7]. The PRCGVPD region was not released, obviously as a result of the matrix-arrested enzyme that was not free to continue the intermolecular process of proteolytic activation in the medium. Thus, cleavage of the Phe^{79}–Met^{80} or the Met^{80}–Leu^{81} peptide bond was not observed at the covalently bound enzyme. Overnight incubation (20 h) yielded very little C-terminally truncated 43 kDa collagenase (Fig. 3), which shared the Met^{65} N terminus with the whole enzyme and had lost the two cysteines of the hemopexin-like domain. This confirms that the propeptide cysteine alone is responsible for interaction with the affinity resin and that the two other cysteine residues play no role in this process. The occurrence of small amounts of the truncated form may be explained as minor side product formed by autolysis on the resin during the long incubation time.

Therefore, our results give clear evidence for a covalent Hg-Cys^{71} interaction during activation. Removal of this residue from its assumed zinc chelating position is, according to the cysteine-switch hypothesis, obviously essential for activity and the following events of propeptide domain cleavages.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft, special research program SFB 223, project B2. The authors wish to thank N. Balke and S. Rottmann for skilful technical assistance and G. Delany for linguistic advice.

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